DiscoverX

User Manual

PathHunter®

β-Arrestin2 Retroparticles

Retroviral Particles to Generate β-Arrestin2 Cell Lines

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Please read entire booklet before proceeding with the assay.
For additional information or Technical Support see contact information below.

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Overview

The PathHunter β-Arrestin2 Retroparticles includes Moloney murine leukemia retrovirus (MMLV) expressing β-arrestin2 fused to the β-galactosidase (β-gal) reporter fragment called EA (enzyme acceptor). The Retroparticles can be used for effective transduction of a wide variety of dividing target cells (e.g. CHO, HEK 293, U2OS) resulting in high-level expression of the encoded β-Arrestin2-EA fusion protein. This product is intended for use with a plasmid or virus expressing a ProLink™-tagged GPCR (or any β-arrestin binding protein) to create assays that report on β-arrestin binding to a target protein of interest or ligand-induced β-arrestin2 recruitment to the cytoplasmic tail of the GPCR of interest. When using this product, we highly recommend that users possess a working knowledge of virus handling and tissue culture techniques.

Product Highlights

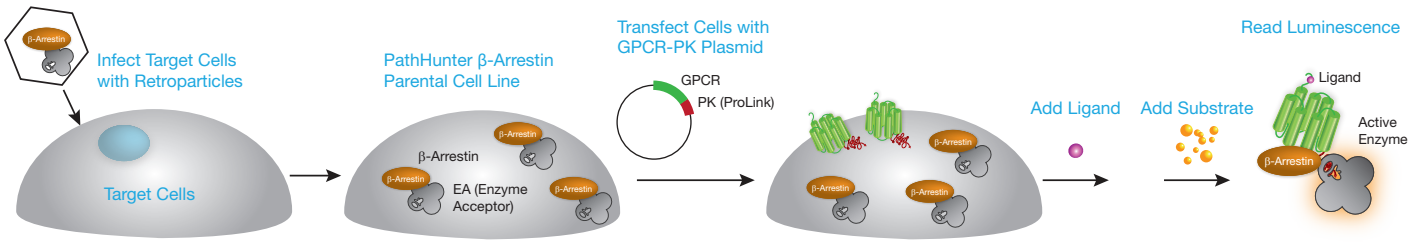
- High levels of β-Arrestin2-EA expression in less than 2 weeks from cell plating through selection
- Retroviral transduction allows for genetically stable, long-term expression of β-Arrestin2-EA fusion protein
- Powerful CMV promoter and VSV-G viral coat that allow use in most cell types
- Replication incompetent retrovirus that is helper virus free
- Most common viral gene delivery system

Technology Principle

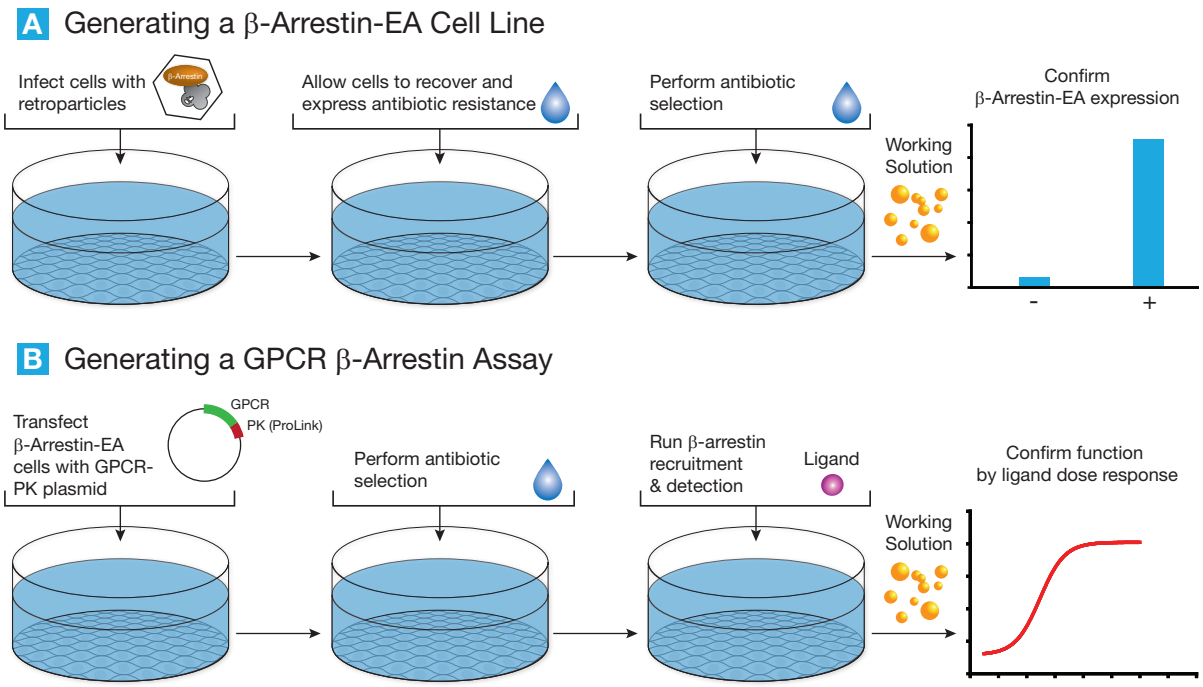
The expression-ready PathHunter β-Arrestin2 Retroparticles are packaged with single stranded (+) mRNA that encodes a hygromycin resistance selection marker and a large β–galactosidase (β-gal) enzyme fragment called the enzyme acceptor (EA) that is fused to the scaffold protein β-arrestin2. Arrestin proteins participate in agonist-mediated desensitization of G-protein-coupled receptors (GPCRs), resulting in dampening of cellular responses to various stimuli, such as hormones or neurotransmitters. β-arrestin regulation has also been described in the literature for other signaling molecules including the protein kinases ERK, JNK, Akt, and PI3 kinase. With the addition of a ProLink-tagged β-arrestin binding protein (i.e. GPCR), you can take advantage of DiscoverX's proprietary Enzyme Fragment Complementation technology to ultimately create your own β-arrestin cell-based assay.

For GPCR applications, this product is designed to evaluate ligand-induced arrestin recruitment to any GPCRs of interest. The GPCR of interest must first be fused to the enzyme donor, ProLink (PK or ED), a smaller β-gal fragment. When the PK-GPCR fusion plasmid is stably co-expressed with the cell line, cells stimulated with the appropriate ligand will induce recruitment of β-Arrestin2-EA to the C-terminal tail of the PK-tagged GPCR. This brings both fragments (EA and PK) of β-gal into close enough proximity to reconstitute a fully functional active enzyme capable of hydrolyzing a substrate molecule and generating a chemiluminescent signal that can be read on any standard luminometer.

Make Your Own GPCR β-Arrestin Cell-Based Assays



Easy-to-Follow Protocols with Readout on Any Standard Luminometer



A. Generate stable PathHunter β-arrestin cell lines that express β-arrestin-EA fusion proteins; and B. Develop a GPCR β-arrestin assay with your GPCR of interest.

Materials Provided

Cat. No.	List of Components	Quantity
30-561	**PathHunter β-Arrestin2-EA Retroparticles**	4 vials of 0.5 mL media containing retroviral particles (Minimum concentration: 1 x 10^6 virus particles/mL)
93-1096S	**PathHunter EA Cell Line Confirmation kit**	
	ED Reagent	1.3 mL
	Cell Lysis Buffer	1.3 mL
	Substrate Reagent	4.5 mL
	EA Positive Control	180 µL

Storage Conditions

PathHunter β-Arrestin2-EA Retroparticles

Store at -80 °C. For single use only. Do not freeze-thaw.

PathHunter EA Cell Line Confirmation kit

Upon receipt, store reagents at -20°C. Thaw reagents at room temperature before use. The reagents can tolerate up to three freeze-thaw cycles with no impact on performance.

Additional Materials Required

Materials		Ordering Information
PathHunter Detection Kit*		93-0001
ProLink™ Vector ** (choose one from the options shown)	pCMV-ProLink Cloning Vector Bundle (includes all 4 PK vectors)	93-0491
	pCMV-ProLink 1 Vector,	93-0167
	pCMV-ProLink 2 Vector,	93-0171
	pCMV-ARMS1-ProLink 2 Vector, or pCMV-ARMS2-ProLink 2 Vector	93-0489 or 93-0490
AssayComplete™ Cell Detachment Reagent		92-0009

* The PathHunter Detection Kit is required for the detection steps of a GPCR functional assay (i.e. ligand response testing).

** A ProLink (PK) vector, also called the enzyme donor (ED), is needed to create a GPCR-PK fusion plasmid that is needed for the Generating a GPCR β-Arrestin2 Cell Line protocol as well.

Note: only one PK cloning vector is required for tagging your specific GPCR. Refer to the GPCR ProLink Vector Guide in the Supplemental Information section for the suggested PK vector based on your GPCR of interest.

Recommended Materials	Ordering Information
PathHunter Anti-EA Antibody	93-0246
AssayComplete Thawing Reagent	92-0016RM Series*
AssayComplete Freezing Reagent	92-0017FR Series*
pCMV-CCR5-PK Expression Vector	93-0224V
Recombinant Human CCL3	92-1002
AssayComplete Hygromycin B	92-0029
AssayComplete G418	92-0030
96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	92-0014
384-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	92-0013
PBS (Dulbecco's Phosphate Buffered Saline Without Calcium and Magnesium)	Corning cellgro Cat. No. 21-031-CM or similar
Multimode or Luminescence Reader	discoverx.com/instrument-compatibility
Single and Multichannel Micro-pipettors and Pipette Tips (10 µL–100 µL)	
Tissue Culture Disposables and Plasticware (10 cm and 6-well cell culture dishes), Polypropylene Conical Tubes (15 or 50 mL), and Tissue Culture Flasks (T25 and T75 flasks, etc.)	
Hemocytometer or Other Cell Counting Device	
Single and Multichannel Micro-pipettors and Pipette Tips (10 µL–1000 µL)	
Cryogenic Vials for Freezing Cells	

*Series refer to the different sizes available for that reagent or kit.

Safety and Handling Conditions

When working with live retroviral particles, follow the recommended federal and institutional guidelines for working with BSL-2 organisms.

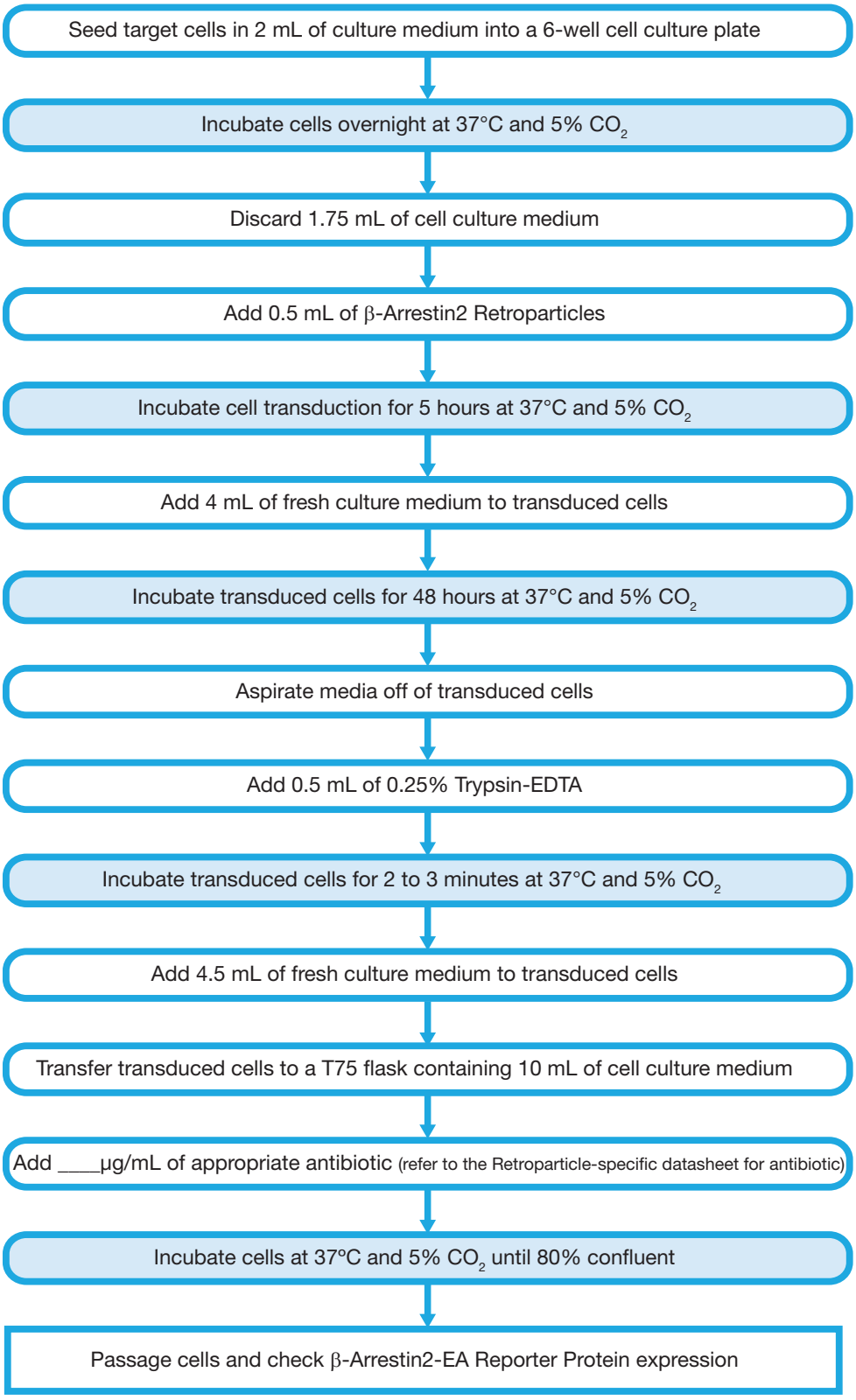
- Perform all manipulations within a certified biosafety cabinet.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.
- Dispose of used pipettes, pipette tips, and other tissue culture supplies as biohazardous waste in accordance with your federal and institutional guidelines.

Strictly follow all published Biosafety Level 2 (BSL-2) safety guidelines when working with retroviral particles with proper waste decontamination. For more information about the BSL-2 guidelines and retrovirus handling, refer to the document, Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded at US National Institutes of Health website at bmbl.od.nih.gov.

Handle all retroviruses in compliance with established institutional guidelines. Since safety requirements for use and handling of retroviral particles may vary at individual institutions, we recommend consulting the health and safety guidelines and/or officers at your institution prior to use of the DiscoverX Retroparticles.

Protocol Schematic: Generating β-Arrestin-EA Parental Cell Lines

Quick-Start Procedure: Each experiment requires a 6-well cell culture plate for each cell type and a T75 cell culture flask:



Detailed Protocol: Generating β-Arrestin-EA Parental Cell Lines

The following is a detailed protocol for infecting an adherent cell host with β-Arrestin2 Retroparticles. Transduction of suspension cell hosts require some modifications to the following protocol. If using a suspension cell line, please refer to the Spin Infection Protocol (modification to the following protocol) described in the Supplemental Information section.

Section I: Target Cell Preparation

The following is a protocol for harvesting target cells and preparing them for plating in a 6-well tissue culture plate, prior to their infection with the Retroparticles. The cells are seeded the day before the Retroparticle transduction. The cell culture medium will depend on the cell line being used. Consult the technical information from the vendor of the cell host being used to determine appropriate cell culture conditions. The protocol assumes that the target cell host is beingcultured in a T75 flask.

1. Pre-warm cell culture medium chosen for culturing the target cell host in a 37°C water bath for 15 minutes.
2. Remove the T75 flask of target cells from the tissue culture incubator and place in a sterile tissue culture hood.
3. Gently aspirate medium from the T75 flask.
4. Add 5 mL PBS into the T75 flask, very gently tip the flask side to side allowing PBS to cover the entire face of the flask to rinse the cells.
5. Gently aspirate PBS from flask.
6. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.
7. Gently rock the flask back and forth to ensure the surface of the flask is thoroughly covered with Trypsin-EDTA.

Prolonged treatment with Trypsin-EDTA may compromise cell viability.

8. Incubate the flask at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
9. Remove the flask from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells do not detach, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.
10. Add 9 mL of cell culture medium to the T75 flask.
11. Using a pipette, gently rinse the cells from the surface of the flask with the added medium.

Keep cells on ice to maintain cell viability until ready for transfer to the 6-well plate.

12. Remove the entire volume of cell suspension from the flask and transfer to a 15 mL conical tube.

Section II: Target Cell Plating

An appropriate number of cells must be added to a well of a 6-well plate. The required cell density on the plate will depend on the cell host being used. At the point the infection is initiated, a target confluency of 40-60% is desired so that the cells will be in log phase growth during the infection incubation period. To reach this target confluency, an appropriate number of cells per well (6-well plate) for a given cell line must be determined empirically. The table below provides examples of suggested cell numbers per well for a variety of common cell lines.

Target Cell Line	Cell Density (per Well)
U2OS	100,000
A549	100,000
HEK 293	400,000
CHO-K1	100,000

The following is a protocol for adjusting the density of the cell suspension and adding 2 mL of cell suspension into wells of a 6-well tissue culture plate.

1. For the purpose of determining the concentration of cells in the suspension,
    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.
    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or other cell counting device.
    c. Count cells and calculate the concentration of cells in the suspension. Then calculate the total number of cells remaining in the 15 mL centrifuge tube.
2. Centrifuge the collected cells at 300 X g for 4 minutes.
3. After centrifugation, carefully remove the supernatant.
4. Re-suspend the cell pellet in pre-warmed (37°C) cell culture medium. Based on the cell number obtained in Step 1 above, add enough cell culture medium so that the cells are suspended at the desired concentration (e.g. 100,000 cells/mL).
5. Gently mix the cells with a pipette so that the cell pellet is completely resuspended.
6. Transfer 2 mL of the cell suspension to one well of the 6-well plate. There should be one well plated with cells for each infection that will be performed.
7. Incubate the 6-well plate overnight at 37°C and 5% CO_2.
8. Proceed to the Retroparticle Infection section.

Section III: Retroparticle Infection

The following is a protocol for transferring Retroparticles to target cells that were plated in a 6-well plate the previous day. One vial of β-Arrestin2 Retroparticles will be used per well of target cells on the 6-well plate. If using a suspension cell line, please refer to the Spin Infection Protocol described in the Supplemental Information section. Some adherent cell hosts, particularly those with slow doubling times, can be prone to low infection efficiencies. The Spin Infection Protocol can also be used with adherent cells to improve infection efficiency, if necessary.

1. Remove one vial of β-Arrestin2 Retroparticles from the -80°C freezer and allow it to thaw at room temperature for 10 minutes or until completely thawed.

Do not thaw Retroparticles in a 37°C water bath, and do not re-freeze them.

2. Retrieve the 6-well cell culture plate containing the target cells from the tissue culture incubator.

After infection, treat spent media containing viral particles with bleach to destroy any live virus.

3. Remove and discard 1.75 mL of medium well. Be careful not to disturb the cells.
4. With a pipette, carefully transfer the full 0.5 mL of the thawed β-Arrestin2 Retroparticles from the vial directly to a single well of target cells. Do not distribute the contents of a single vial of Retroparticles across multiple wells with target cells. This will adversely impact the infection efficiency.
5. Return the 6-well plate to the tissue culture incubator and incubate at 37°C and 5% CO_2 for 5 hours.

Section IV: Cell Recovery

The following is a protocol for initiating target cell recovery.

1. Remove the 6-well cell culture plate containing infected cells from the incubator.
2. Add 4 mL of fresh cell culture medium to the wells containing the infected cells.
3. Incubate plate at 37°C and 5% CO_2 for 48 hours.

Section V: Antibiotic Selection

The β-Arrestin2 Retroparticles contain an antibiotic resistance marker to enable positive selection. The antibiotic concentration required to generate a stable pool are dependent on the cell host being used, and must be determined empirically. It is recommended that a kill curve be generated to determine the antibiotic concentration required to kill non-transduced cells. The appropriate concentration of antibiotic must be determined before the infection experiment is begun.

Recommended Hygromycin B Concentration

Target Cell Line	Hygromycin B (µg/mL)
U2OS	250
A549	300
HEK 293	200
CHO-K1	300

The table to the left contains recommended concentrations of Hygromycin B for several commonly used cell lines.

At this point, the infection is complete. The following is a protocol for initiating antibiotic selection of the infected cells to generate a stable pool.

1. Pre-warm cell culture medium in a 37°C water bath for 15 minutes.
2. Remove the 6-well plate from the tissue culture incubator and place in a sterile tissue culture hood.
3. Gently aspirate media from the infected cells in the 6-well plate.
4. Add 2 mL PBS into the well, very gently tip the 6-well plate from side-to-side to rinse cells.
5. Gently aspirate PBS from the well.
6. Add 0.5 mL of 0.25% Trypsin-EDTA to the well.
7. Gently rock the 6-well plate back and forth to ensure the surface of the well is thoroughly covered with Trypsin-EDTA.
8. Incubate the plate at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
9. Remove the plate from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the plate to detach cells from the surface. If the cells do not detach, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.
10. Add 4.5 mL of cell culture medium to the well.
11. Using a pipette, gently rinse the cells from the surface of the well with the added medium.

Prolonged treatment with Trypsin-EDTA may compromise cell viability.

12. Transfer 5 mL of cells to a clean T75 tissue culture flask.
13. Add 10 mL of cell culture medium to the T75 flask. The total volume in the flask now should be 15 mL.
14. Add an appropriate volume of selection antibiotic to the flask to achieve the predetermined (e.g determined from a kill curve) concentration.
15. To generate a stable pool overexpressing the β-Arrestin2-EA Reporter Protein, incubate plate at 37°C and 5% CO_2 in a humidified tissue culture incubator until cells reach 70-80% confluency (typically 5 to 10 days).
16. To ensure the complete antibiotic selection has been achieved, passage cells one time using a conservative (i.e 1:2 or 1:3) cell split ratio.
    a. Pre-warm cell culture medium supplemented with the pre-determined concentration of selection antibiotic in a 37°C water bath for 15 minutes
    b. Remove the T75 flask from the tissue culture incubator and place in a sterile tissue culture hood.
    c. Gently aspirate media from the T75 flask.
    d. Add 5 mL PBS into the T75 flask, very gently tip the flask side-to-side allowing PBS to cover the entire face of the flask to rinse the cells.
    e. Gently aspirate PBS from flask.

f. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.

g. Gently rock the flask back and forth to ensure the surface of the flask is thoroughly covered with Trypsin-EDTA.

h. Incubate the flask at 37°C and 5% CO_2 for 2 to 4 minutes or until the cells have detached.

Prolonged treatment with Trypsin-EDTA may compromise cell viability.

i. Remove the flask from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells do not detach, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.

j. Add 4 mL of the cell culture media to the T75 flask.

k. Transfer one half to one third (depending on whether or not a 1:2 or 1:3 split ratio is used) of the cell suspension from the first flask to the 12 mL of media in the new T75 flask. Transfer flask to a tissue culture incubator and incubate cells until they reach 70-80% confluency at 37°C and 5% CO_2.

17. Incubate T75 flask at 37°C and 5% CO_2 in a humidified tissue culture incubator until cells reach 70-80% confluency.

18. It's recommended that the β-Arrestin2-EA parental cell pool be cryopreserved for long-term storage. Refer to the Cell Freezing protocol in the Supplemental Information section.

19. Proceed to Detection of Expression Level.

Section VI: Detection of Expression Level

By this point, the cell pool should be stably expressing the β-Arrestin2-EA Reporter Protein. The following is a protocol for evaluating the expression level of the β-Arrestin2-EA Reporter Protein in this stable pool using the PathHunter EA Cell Line Confirmation Kit (Cat. No. 93-1069S). Cells will be treated with solutions containing cell lysis buffer and either ED Reagent or buffer. The differences of signal generated by the two treatments (with vs. without ED Reagent) will reveal expression of the β-Arrestin2-EA Reporter Protein. It can also be informative to simultaneously run this experiment on the native parental cells used to generate the β-Arrestin2 cell pool, to compare the basal activities of the transduced cells versus the non-transduced cells. The expression confirmation kit also contains an EA Control reagent for use as a detection positive control.

Alternatively, expression can be detected via Western blot analysis using the PathHunter Anti-EA Antibody (Cat. No. 93-0246).

Although testing for expression of the β-Arrestin2-EA Reporter Protein is recommended, a third (indirect) option for assessing β-Arrestin2-EA expression would be to generate a functional GPCR test cell pool, using the pCMV-CCR5-PK Expression Vector (Cat. No. 93-0224V) as a control. The resulting pool can be tested for a functional response to an agonist of CCR5. Refer to Detailed Protocol: Generating a GPCR β-Arrestin2 Assay for instructions for generating and running a functional GPCR Arrestin recruitment assay.

1. Remove the T75 flask containing the β-Arrestin2 stable pool from the tissue culture incubator.

2. Aspirate the medium from the flask.

3. Add 5 mL PBS into the T75 flask and very gently tip the flask side-to-side to allow the PBS to cover the entire face of the flask to rinse the cells.

4. Gently aspirate the PBS from the flask.

5. Add 1 mL of 0.25% Trypsin-EDTA to the T75 flask.

6. Gently rock the flask back and forth to ensure the surface of the flask is thoroughly covered with Trypsin-EDTA.

7. Incubate the flask at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached. Remove the flask from the incubator and confirm the cells are detached by viewing under a microscope. If necessary, gently tap the edge of the flask to detach cells from the surface. If the cells do not detach, return to the incubator for an additional 1 to 2 minutes and repeat this step until cells are in suspension.

   Prolonged treatment with Trypsin-EDTA may compromise cell viability.

8. Add 9 mL of cell culture medium to the T75 flask. If also testing non-transduced native cells for their basal activity, follow the same steps for harvesting the cells.

9. Using a pipette, gently rinse the cells from the surface of the flask with the added medium.

10. Remove the entire amount of cells from the flask and transfer to a 15 mL conical centrifuge tube.

11. For the purpose of determining the concentration of cells in the suspension:

    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.

    Keep suspended cells on ice to maintain cell viability until ready for transfer to the assay plate.

    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or other cell counting device.

    c. Count cells and calculate the concentration of cells in the suspension. Then calculate the total number of cells remaining in the 15 mL centrifuge tube.

12. Based on the cell number obtained in Step 11 above, dilute the resuspended cells to 125,000 cells/mL (i.e. 2,500 cells/20 µL).

13. Transfer a portion of the cells to a sterile reservoir.

14. Transfer 20 µL (2,500 cells) of the cell suspension to each of 8 wells of a 384-well assay plate. If also testing non-transduced cells, be sure to plate them in wells other than the 8 wells occupied by the β-Arrestin2 cell pool.

    Be sure to save a portion of the cell suspension for propagating the β-arrestin2 pool in a separate tissue culture flask.

15. For EA Positive Control wells: Prepare 1:4 dilution of the EA Positive Control by mixing 1-part of the EA Positive Control reagent with 3-parts of PBS. For example, mix 45 µL of EA Positive Control reagent with 135 µL PBS in a tube. Transfer 20 µL of the 1:4 dilution of the EA Positive Control to each of 8 empty (i.e. no cells) wells on the 384-well assay plate.

16. Transfer the assay plate to a tissue culture incubator to incubate the assay plate at 37°C and 5% CO_2 until EA Confirmation detection solutions are prepared and ready to be added to the cells.

17. Prepare two working EA Confirmation detection solutions in separate tubes: one solution with ED Reagent (for positive signal detection); the second solution with PBS (for negative control). The two working solutions consist of 4-parts of Substrate Reagent, 1-part of ED Reagent (or PBS for the negative control), and 1-part of Cell Lysis Buffer. If testing non-transduced native cells, increase by 50% the reagent volumes indicated in the table below.

    Working EA Confirmation detection solution is light sensitive, thus incubation in the dark is necessary.

Assay Reagents	Working EA Confirmation Detection Solutions - With ED Reagent	Working EA Confirmation Detection Solutions - Without ED Reagent
Substrate Reagent (µL)	200	200
ED Reagent (µL)	50	0
Cell Lysis Buffer (µL)	50	50
PBS (µL)	0	50
Total Volume (µL)	**300**	**300**

18. Mix reagents by gently inverting the tubes a few times. Briefly centrifuge the tubes to make sure the entire reagent volume is at the bottom of the tube.

19. Remove the 384-well assay plate from the tissue culture incubator.

20. To each of four wells with β-Arrestin2 parental cells, add 30 µL of working EA Confirmation detection solution containing ED Reagent. To each of the other four wells, add 30 µL of EA Confirmation detection solution containing PBS. Also add the two working detection solutions to wells (4 replicates each) with the EA Positive Control, and with the (optional) native cell control. It is not recommended that the wells be mixed by pipetting up and down or by vortexing the plate.

21. Place lid back on plate and incubate assay plate for at least 30 minutes at room temperature in the dark.

22. Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for imager. The actual signal characteristics over time are affected by lab conditions, such as temperature, and the user should establish an optimal read time. In general, the signal continues to increase. The plate may be incubated overnight (16 hours) and the signal may be measured the next day. Once an optimal read time has been established, continue to use this incubation time to maintain consistency between assays. Luminescence readout usually collects signal from all wavelengths. Some instrument manufacturer may include a cutoff filter at high wavelengths, but usually no wavelength setting is needed for luminescence readout.

23. The signal/background ratio must be calculated to evaluate the relative expression of the β-Arrestin2-EA Reporter Protein.

    a. Calculate the mean of the RLU values generated by the 4 replicate wells treated with the working EA Confirmation detection solution containing ED Reagent. The result is the **Mean Signal**.

    b. Calculate the mean of the RLU values generated by the other 4 replicate wells treated with the working solution containing PBS. The result is the **Mean Background**.

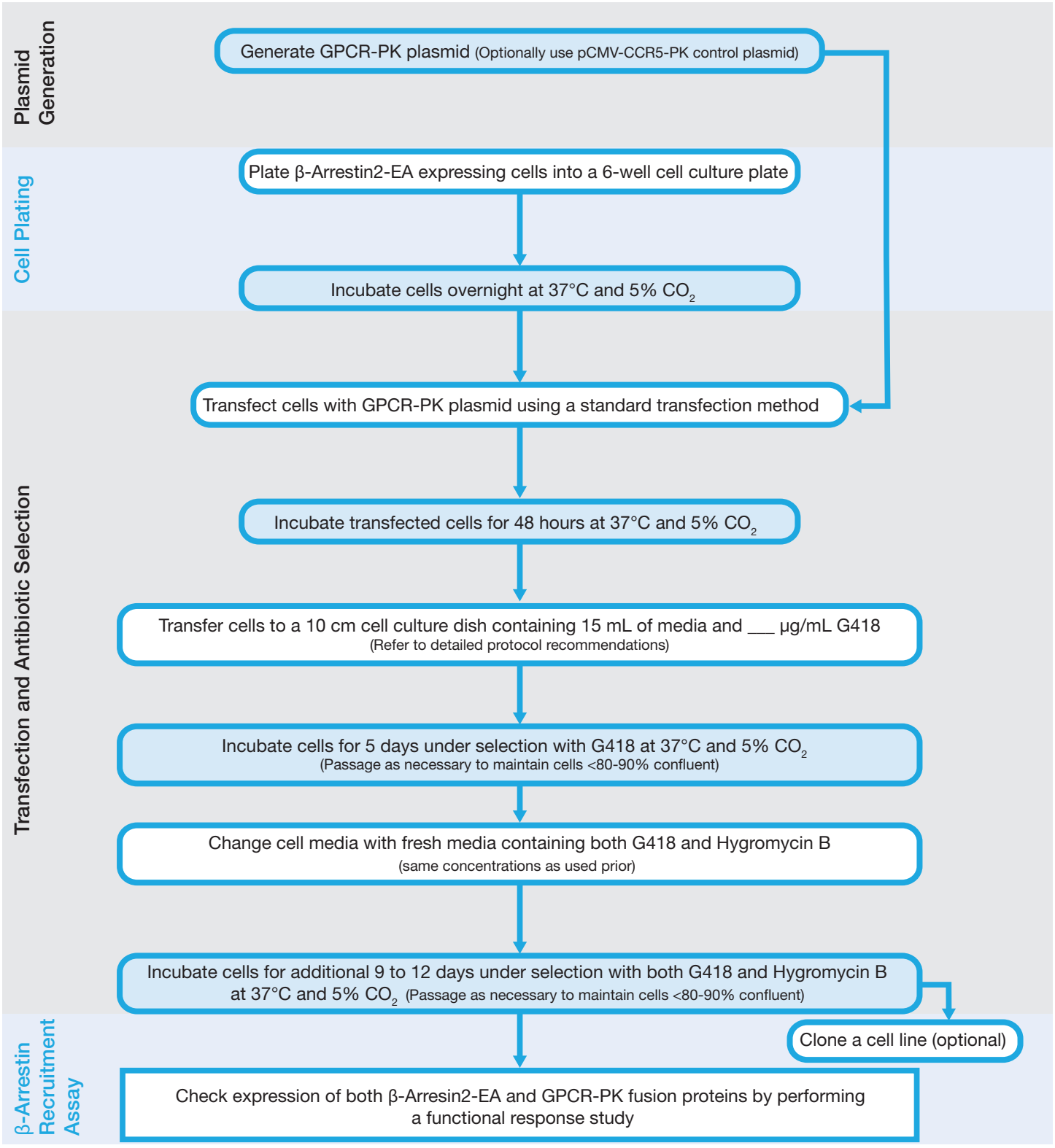
    c. Divide the **Mean Signal** by the **Mean Background**. The result is the Signal/Background Ratio (S/B ratio).

    S/B ratio = Mean Signal / Mean Background

24. Refer to Guidelines for Interpreting Expression Results (β-Arrestin2-EA) in the Supplemental Information section.

25. If selection of a clonal parental cell line is desired, refer to Generating a Stable Clone (Optional) in the Supplemental Information section.

Protocol Schematic: Generating a GPCR β-Arrestin2 Assay



Detailed Protocol: Generating a GPCR β-Arrestin2 Assay

By this point, the β-Arrestin2-EA parental cell pool has been generated. The following is a detailed protocol for transfecting a plasmid containing a ProLink™ (PK) tagged GPCR into the β-Arrestin2-EA cell line, and generating a clonal PathHunter GPCR β-Arrestin2 recruitment cell line.

Section I: Plasmid Generation - Construction of a Vector with a ProLink-Tagged GPCR

When generating a stable GPCR β-arrestin2 cell line, a plasmid vector containing a ProLink-tagged GPCR construct is required. GPCR-PK vectors can be constructed by cloning the GPCR of interest into one or more of several available pCMV-ProLink expression vectors (refer to the table below for a list of available vectors) or purchase a GPCR-PK vector from the DiscoverX catalogue. The DiscoverX pCMV-CCR5-PK Expression Vector (Cat. No. 93-0224V) is offered for generating a positive control cell line. The GPCR-PK plasmid vector is then transfected into the β-Arrestin2-EA parental cells.

If generating a GPCR-PK plasmid, please refer to the tables in the Supplemental Information section for recommendations on which PK expression vector to use for many GPCRs. Alternatively, you may choose to test all four PK tags that are available, and empirically determine the tag that results in an assay with the best dynamic range. PK1 is the most commonly used ProLink tag for PathHunter β-Arrestin2 cell lines.

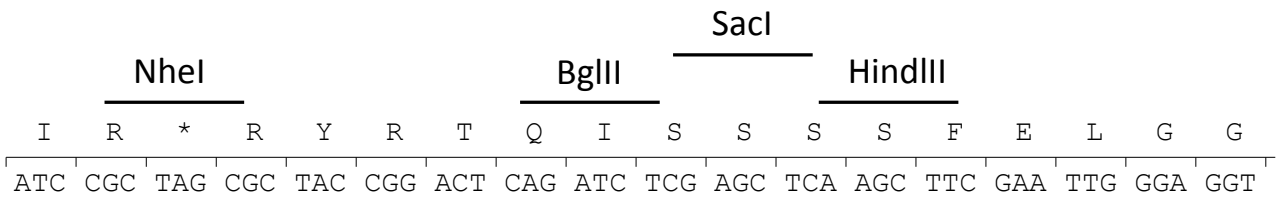
ProLink Vectors			
Vector Name	**Cat. No.**	**Vector Features**	**Application**
pCMV-ProLink 1	93-0167	Low Affinity PK1 tag	Standard protein:protein interaction
pCMV-ProLink 2	93-0171	High Affinity PK2 tag	Weak protein:protein interaction
pCMV-ARMS1-ProLink 2*	93-0489	High affinity PK2 tag, plus ARMS1	Enhance β-arrestin recruitment
pCMV-ARMS2-ProLink 2*	93-0490	High affinity PK2 tag, plus ARMS2	Enhance β-arrestin recruitment

* ARMS (Arrestin Recruitment Modulating Sequence) is an 18-21 amino acid spacer between the GPCR and the PK tag that has been shown to enhance β-arrestin recruitment. There are two variants of the ARMS spacer: ARMS1 and ARMS2.

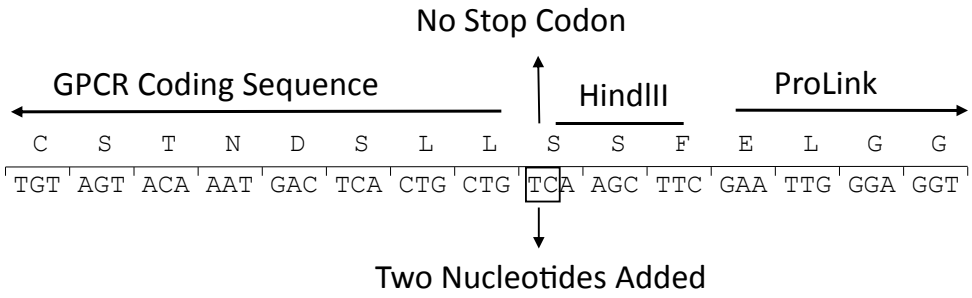
ProLink Vector Cloning Information

pCMV-ProLink vectors contain the PK tag, a 4 kDa fragment of β-gal that has with a low affinity for the larger EA β-gal fragment. All vectors utilize a CMV promoter for expression in mammalian cells. When properly constructed, a GPCR fusion protein, with the PK tag on the C-terminus of the GPCR, should result. The pCMV-ProLink vector sequences do not contain a start codon, and thus the presence of a start codon and a Kozak sequence in the GPCR gene of interest is required. To enhance expression, it's recommended that ACC (the Kozak nucleotide sequence) be positioned immediately prior to the ATG start codon. The GPCR must be in frame with the PK peptide and must not contain a stop codon anywhere in the receptor's nucleotide sequence. Due to the positioning of available restriction sites in the polylinker, it may be necessary to add additional nucleotides (between the GPCR, and the ProLink tag) to maintain integrity of the PK tag's reading frame. Refer to the figure (on page 15) representing an example of what would be required for sub-cloning a receptor into the Hind III site. Use 50 µg/mL Kanamycin for propagating plasmid DNA in E. coli cells.

Multiple Cloning Site Sequence



Example of a GPCR Properly Inserted In-Frame with the PK Tag



When designing a PCR primer for the 3' end of the GPCR, the GPCR stop codon should be removed. Primers must also be designed for in-frame fusion with PK. For example, when using the Hind III site, add two nucleotides before the AAGCTT to ensure that the reading frame is maintained between the GPCR and PK. Other restriction sites require nucleotide additions as well to maintain reading frame integrity.

Use of the pCMV-CCR5-PK Expression Vector (Optional)

The pCMV-CCR5-PK Expression Vector (Cat. No. 93-0224V) is the recommended plasmid for generating a positive control cell line. The plasmid encodes human C-C motif chemokine receptor 5 (CCR5; BC038398.1), with the PK sequence fused to the C-terminus of the protein. The product comes with 10 µg of pCMV-CCR5-PK plasmid DNA and the datasheet includes a vector map. To propagate, transform plasmid DNA into E. coli cells using 50 mg/mL Kanamycin as a selection marker.

The control agonist for functionally testing a CCR5 β-arrestin2 cell line is Recombinant Human CCL3 (MIP-1α; Cat. No. 92-1003). Refer to the ligand datasheet for additional details regarding this control agonist.

Section II: Cell Plating

The following is a protocol for plating β-arrestin2-EA parental cells in preparation for transfection of the GPCR-PK plasmid. The transfection protocol to follow assumes that transfection of the plasmid will be accomplished using a lipid transfection reagent.

1. Plate 100,000 to 400,000 β-Arrestin2-EA parental cells with 2 mL of cell culture medium, in a well of a 6-well cell culture plate. An optimal cell number for transfection with a lipid-based transfection reagent should be determined empirically. It's recommended that there be wells set up for the CCR5-PK control plasmid as well as for the GPCR of interest.
2. Incubate cells overnight at 37°C and 5% CO_2. Suspension cells can be plated and transfected the same day.
3. Proceed to Transfection and Stable Pool Selection.

Section III: Transfection and Stable Pool Selection

The following is a protocol for setting up a transfection of a GPCR-PK plasmid vector into the β-Arrestin2-EA parental cells, then placing the cells under antibiotic selection to generate a stable pool. The protocol assumes that vector delivery will be accomplished using a lipid transfection reagent selected by the user. If using other transfection methods, such as nucleofection, follow the manufacturer's recommended transfection protocol. Transfection efficiency can be evaluated by transfecting cells with a plasmid containing a fluorescent reporter gene. Refer to Determination of Transfection Efficiency (Optional) in the Supplemental Information section for details.

1. Follow the recommended protocol from the manufacturer of the lipid transfection reagent of choice to design an appropriate transfection mix and transfection protocol.
2. Prepare separate transfection mixes with the pCMV-GPCR-PK plasmid of interest, and the pCMV-CCR5-PK control vector (if used).
3. Add the transfection mix to the β-Arrestin2-EA parental cells in the 6-well tissue culture plate.
4. Incubate transfected cells for 48 hours at 37°C and 5% CO_2.
5. Initiate antibiotic selection by detaching the transfected cells from the 6 well plate and transfer them to a T75 containing 15 mL (final volume) of medium supplemented with G418. Refer to the table to the right for recommended G418 concentrations for common target cell lines. If your target cell line is not indicated, perform a kill curve prior to initiating the transfection step to determine the optimal concentration of G418 to use for selection.

Recommended G418 Concentrations	
Target Cell Line	**Final G418 Concentration (µg/mL)**
U2OS	500
HEK 293	800
CHO-K1	800
A549	500

6. Incubate cells for about 5 days (or until healthy colonies are seen) under antibiotic selection with G418 at 37°C and 5% CO_2.
7. Carefully remove and replace the G418 selection media with fresh selection media containing G418 and Hygromycin B.
8. Incubate cells for an additional 9 to 12 days under antibiotic selection with both G418 and Hygromycin B at 37°C and 5% CO_2. Cells should be passaged as needed, but conservatively (i.e. 1:2-1:3 split ratios) to maintain cells in log phase growth. It's recommended that cells be passaged at least 1-2 times, to ensure that selection the pool of cells is stably expressing both the β-Arrestin2-EA and GPCR-PK fusion proteins.
9. When selection of the stable pool is complete, and cells have reached 70-80% confluency, it's recommended that the PathHunter GPCR β-Arrestin2-EA stable pool be cryopreserved for long-term storage. Refer to the Cell Freezing protocol in the Supplemental Information section.

Section IV: β-Arrestin Recruitment Assays

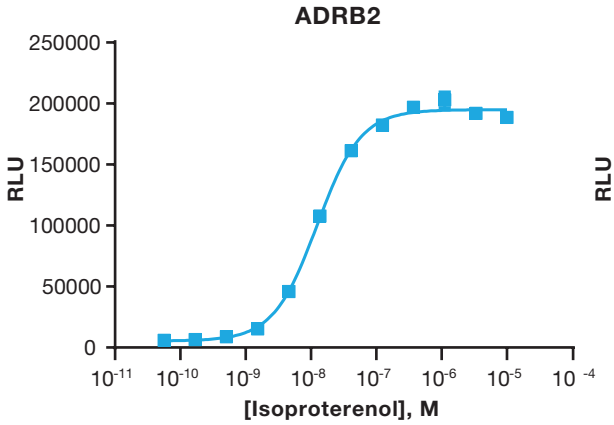
By this point, the stable cell pool is ready for functional testing. Cells should be tested for a dose-dependent response to an appropriate receptor agonist. Manuals containing detailed protocols for running PathHunter β-Arrestin recruitment assays using PathHunter detection reagents are available on the DiscoverX website. Refer to Protocols for Running PathHunter β-Arrestin2 Recruitment Assays in the Supplemental Information section for web links to the appropriate user manuals, and other details useful for setting up and optimizing the assays. Refer to the Typical Results section for data from two PathHunter β-Arrestin recruitment assays developed by DiscoverX.

If there is no agonist available (i.e. for an orphan GPCR) to use for functional testing, then the stable pool can be tested for expression of the GPCR-PK fusion using the PathHunter ProLabel®/ProLink™ Detection Kit (Cat. No. 93-0812 Series). Refer to Protocols for Detecting Expression of Orphan GPCR-PK in the Supplemental Information section for web links to the user manual for the PathHunter ProLabel/ProLink Detection Kit.

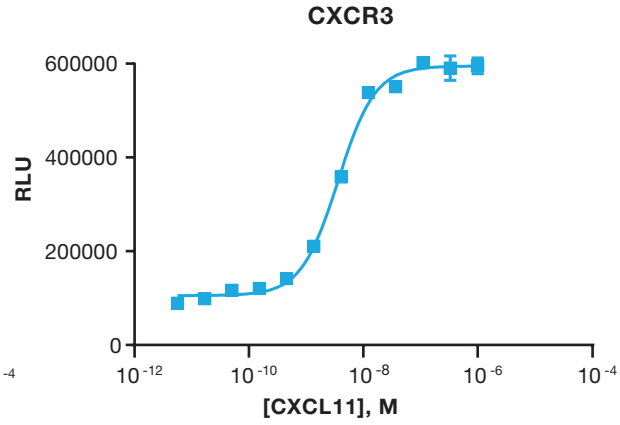
It is recommended that a clonal cell line be isolated from the stable pool. Clonal cell lines possess superior passage stability, and a more robust cell assay may result. If selection of a clonal cell line is desired, refer to Generating a Stable Clone in the Supplemental Information section.

Typical Results

Assay results from PathHunter stable GPCR cell assays expressing the β-Arrestin2-EA Reporter Protein, and either ProLink™-tagged ADRB2 or CXCR3 in CHO-K1 cells. The resulting PathHunter β-Arrestin recruitment assays were induced with the agonists isoproterenol (for ADRB2) or CXCL11 (for CXCR3).



EC_{50}	S/B
12 nM	32.3



EC_{50}	S/B
38 nM	6.7

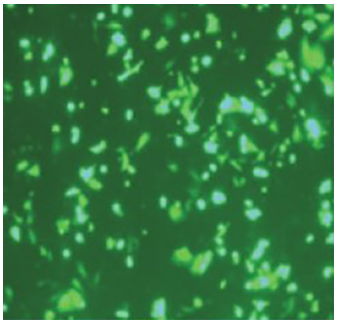
Supplemental Information

Guidelines for Interpreting Expression Results (β-Arrestin2-EA)

Fold expression (the ratio of the signal from the working EA Confirmation detection solution with ED Reagent relative to that without ED Reagent) will typically be >20 for a pool transduced with β-Arrestin2-EA and approximately 1 for un-transduced, native cells (e.g. not expressing β-Arrestin2-EA).

Determination of Transfection Efficiency (Optional)

To determine the percentage of cells that are transfected with DNA, a reporter gene is often used. This step would typically be performed in parallel (in a separate well) with your GPCR-PK transfection. One convenient reporter for monitoring transfection efficiency is the Enhanced Green Fluorescent Protein (EGFP). When excited by UV light, the EGFP protein emits a green fluorescent signal that can be readily detected by microscopy or flow cytometry. The ratio of fluorescent versus non-fluorescent cells determines the overall efficiency of the transfection experiment and can be used to estimate the number of cells that would be expressing your GPCR of interest



PathHunter CHO-K1 Arrestin-EA Parental cells were plated, incubated for 24 hours, then transfected with 2 µg of pEGFP vector. Cells were imaged 24 hours post-tansfection. Transfection efficiency was determined to be greater than 60% based on EGFP expression.

Protocol for Running PathHunter β-Arrestin2 Recruitment Assays

The newly established GPCR β-Arrestin2 cell pool can be used for testing GPCR activity. For detailed protocols for running PathHunter β-Arrestin recruitment assays and for using the PathHunter detection reagents, refer to the following user manuals (located on the DiscoverX website, at the links indicated below) for PathHunter β-Arrestin Assays and the PathHunter Detection Kit (Cat. No. 93-0001 Series).

PathHunter β-Arrestin Assay for GPCR Cell Lines: discoverx.com/ArrestinUM

PathHunter Detection Kit: discoverx.com/PathHunterDetectionUM

PathHunter β-Arrestin assays are typically set in 384-well assay plates by plating 5,000 cells per well; followed by an overnight incubation at 37°C and 5% CO_2 (to allow the cells to attach and grow) before treating the cells with compounds. Assays in 96 well plates typically require at least 10,000 cells per well. However, an optimal cell seeding density should be determined empirically.

Agonist induction periods are typically 90 minutes at 37°C and 5% CO_2. For some GPCR targets, the assay dynamic range can be improved by increasing the agonist induction period and/or by incubating the assay at room temperature. Induction periods of less than 90 minutes are not recommended, as they typically result in lower signal/background ratios. An optimal agonist incubation period, and incubation temperature should be determined empirically.

Antagonist pre-incubation periods are typically 30 minutes at 37°C and 5% CO_2.

Protocol for Detecting Expression of Orphan GPCR-PK

Cell assays for orphan GPCRs may have to be evaluated for expression of the GPCR-PK fusion if no agonist is available for functional testing. Expression of the GPCR-PK fusion can be detected using the PathHunter ProLabel®/ProLink™ Detection Kit (Cat. No. 93-0812 Series). Refer to the following user manual (located on the DiscoverX website, at the link indicated below) for testing expression of a ProLink tagged orphan GPCR:

PathHunter ProLabel/ProLink Detection Kit: discoverx.com/PLPKdetectionUM

GPCR ProLink™ Vector Guide

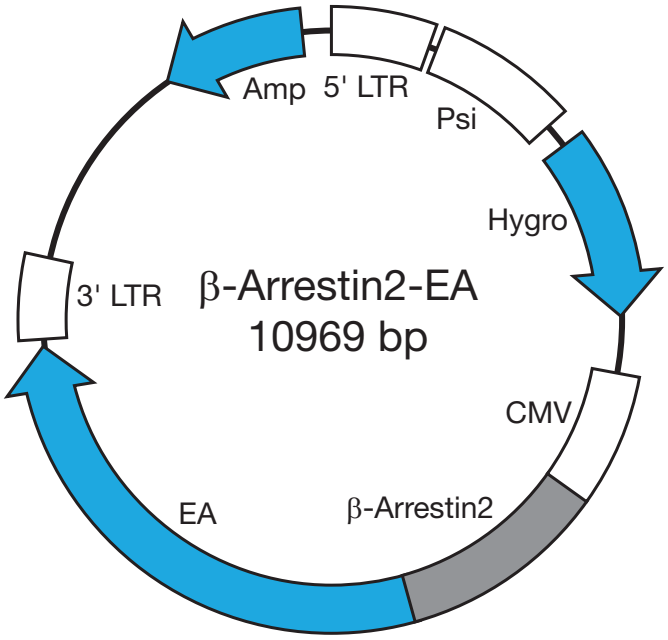
Use the following tables and guidelines to determine the recommended PK vector for your GPCR of interest. For orphan GPCRs, we recommend using PK1 vector. Note ARMS2-PK2 vectors can also be used for the GPCR targets that recommend the ARMS1-PK1 vector.

Human GPCR ProLink Vector Guide

GPCR Gene ID	PK Tag	GPCR Gene ID	PK Tag	GPCR Gene ID	PK Tag	GPCR Gene ID	PK Tag
ADCYAP1R1	PK1	CHRM5	ARMS2-PK2	GLP2R	PK1	NTSR1	PK1
ADORA1	PK1	CMKLR1	PK1	GPR1	PK1	OPRD1	PK1
ADORA2B	PK1	CMKOR1	PK1	GPR109A	PK1	OPRK1	PK1
ADORA3	PK1	CNR1	PK1	GPR119	ARMS2-PK2	OPRL1	PK1
ADORA3	ARMS2-PK2	CNR2	PK1	GPR120	PK1	OPRM1	PK1
ADRA1B	PK1	CRHR1	PK1	GPR35	ARMS2-PK2	OXTR	PK1
ADRA2A	ARMS2-PK2	CRHR2	PK1	GPR55	PK1	P2RY11	PK1
ADRA2B	ARMS2-PK2	CRTH2	PK1	GPR81	PK1	P2RY12	PK1
ADRA2C	ARMS2-PK2	CX3CR1	PK1	GPR84	PK1	P2RY2	PK1
ADRB1	PK1	CXCR1	PK1	GPR91	PK1	P2RY4	PK1
ADRB2	PK1	CXCR2	PK1	GPR92	PK1	P2RY6	ARMS2-PK2
AGTR1	PK1	CXCR3	PK1	GRPR	PK1	PPYR1 (NPY4)	PK1
AGTRL1	PK1	CXCR4	PK1	HCRTR1	PK1	PRLHR	PK1
AVPR1A	PK1	CXCR5	ARMS1-PK2	HCRTR2	PK1	PROKR1	PK1
AVPR1b	PK1	CXCR6	PK1	HRH1	ARMS2-PK2	PROKR2	PK1
AVPR2	PK1	DRD1	PK1	HRH2	PK1	PTAFR	PK1
BDKRB1	ARMS2-PK2	DRD2L	ARMS2-PK2	HRH3	ARMS2-PK2	PTGER2	PK1
BDKRB2	PK1	DRD2S	ARMS2-PK2	HRH4	PK1	PTGER3	ARMS2-PK2
BRS3	ARMS2-PK2	DRD3	ARMS2-PK2	HTR1A	ARMS2-PK2	PTGER3	ARMS2-PK2
C5AR1	PK1	DRD4	ARMS2-PK2	HTR1B	ARMS1-PK1	PTGER4	PK1
C5L2	PK1	DRD5	PK1	HTR1E	ARMS2-PK2	PTGIR	PK1
CALCR	PK1	EDG1	PK1	HTR1F	ARMS2-PK2	PTHR1	PK1
CALCR + RAMP2	PK1	EDG2	PK1	HTR2C	PK1	PTHR2	PK1
CALCR + RAMP3	PK1	EDG3	PK1	HTR5A	ARMS2-PK2	SALPR	ARMS1-PK2
CALCRL + RAMP1	PK1	EDG4	PK1	HTR6	PK1	SCTR	PK1
CALCRL + RAMP2	PK1	EDG5	PK1	KISS1R	PK1	SSTR1	PK1
CALCRL	PK1	EDG6	PK1	LHCGR	PK2	SSTR2	PK1
CCKAR	PK1	EDG7	PK1	LTB4R	PK1	SSTR3	PK1
CCKBR	PK1	EDG8	PK1	MC1R	ARMS1-PK1	SSTR4	ARMS2-PK2
CCR1	PK1	EDNRA	PK1	MC3R	PK2	SSTR5	PK1
CCR10	PK1	EDNRB	PK1	MC4R	PK1	TACR1	PK1
CCR2	PK1	F2R	PK1	MC5R	PK1	TACR2	PK1
CCR3	PK1	F2RL1	PK1	MCHR1	PK1	TACR3	PK1
CCR4	PK1	F2RL3	PK1	MCHR2	ARMS2-PK2	TBXA2R	PK1
CCR5	PK1	FPR1	PK1	MLNR	PK1	TRHR	PK1
CCR6	PK1	FPRL1	PK1	MRGPRX2	PK1	TSHR	PK1
CCR7	PK1	FSHR	ARMS1-PK2	MTNR1A	ARMS1-PK2	UTR2	PK1
CCR8	PK1	GALR1	PK1	MTNR1B	ARMS2-PK2	VIPR1	PK1
CCR9	PK1	GALR2	PK1	NMU1R	PK1	VIPR2	PK1
CHRM1	ARMS1-PK1	GCGR	PK1	NPBWR1	ARMS2-PK2	XCR1	PK2
CHRM2	PK1	GHSR1a	PK1	NPBWR2	ARMS2-PK2		
CHRM3	ARMS1-PK1	GIPR	PK1	NPY1R	PK1		
CHRM4	PK1	GLP1R	PK1	NPY2R	PK1		

Ortholog GPCR ProLink Vector Guide							
GPCR Gene ID	**PK Tag**	**GPCR Gene ID**	**PK Tag**	**GPCR Gene ID**	**PK Tag**	**GPCR Gene ID**	**PK Tag**
cEDG5	PK1	mCCR8	PK1	mEDNRA	PK1	mOXTR1	PK1
mADCYAP1R1	PK1	mCCR9	PK1	mEDNRB	PK2	mP2RY6	ARMS2-PK2
mADCYAP1R1	PK1	mCMKLR1	PK1	mFPR1	PK1	mP2RY12	PK1
mADORA2B	PK1	mCNR1	PK1	mGALR2	PK2	mPPYR1 (mNPY4R)	ARMS2-PK2
mADORA3	PK1	mCNR2	PK1	mGHSR1a	PK1	mPTAFR	PK1
mAGTRL1	PK1	mCRHR1	PK1	mGHSR1a	PK1	mPTAFR	PK1
mBRS3	PK1	mCRTH2	PK1	mGLP1R	PK1	mSSTR2	PK1
mC5AR1	PK1	mCXCR2 (IL8RB)	PK1	mGPR1	PK1	mSSTR5	PK1
mCCKAR	PK1	mCXCR3	PK1	mHTR2A	PK1	mUTR2	PK1
mCCR1	PK1	mCXCR4	PK1	mKISS1R	PK1	mVIPR1	PK1
mCCR2	PK1	mCXCR5	PK1	mLTB4R1	PK1	rCRTH2	PK1
mCCR3	PK1	mCXCR6	PK1	mMCHR1	PK1	rEDG5	PK1
mCCR4	PK1	mCXCR7	PK1	mNPY1R	PK1	rOPRM1	PK1
mCCR5	PK1	mDRD5	PK1	mNPY2R	PK1	rPROKR1	PK1
mCCR6	PK1	mEDG1	PK1	mOPRD1	PK1	rPROKR2	PK1
mCCR6	PK1	mEDG5	PK1	mOPRK1	PK1	sEDG5	PK1
mCCR7	PK1	mEDG6	PK1	mOPRM1	PK1		

β-Arrestin2-EA Vector Map



Spin Infection Protocol (Optional)

Spin infection can augment Retroparticle transduction efficiency for both suspension and adherent cells.

The following spin infection modification to the infection protocol is recommended when infecting adherent cells prone to poor infection efficiency, and for suspension cells lines. Cell lines with slow doubling times can have particularly poor infection rates.

Spin Infection Protocol Section I: Preparing Suspension Cells for Spin Infection

1. Prepare cells for plating in wells of a 6-well tissue culture plate:
    a. For Suspension cells: harvest 1 million suspension cells from their tissue culture flask; transfer them to a 15 mL conical tube; spin them to pellet the cells; carefully aspirate the growth medium; and resuspend the cells in 0.5 mL fresh, complete growth medium. Note: be sure that the cells are in log phase growth before initiating this step.
    b. For Adherent cells: follow Sections I and II in Detailed Protocol: Generating β-Arrestin-EA Parental Cell Lines for preparing, and plating adherent cells in the well of a 6-well tissue culture plate.
2. Transfer the suspended target cells into one well of a 6-well tissue culture plate.
3. For adherent cells, incubate the 6-well plate overnight at 37°C and 5% CO_2 to allow the cells to attach and grow before proceeding to the next section. For suspension cells, proceed to the next section the same day cells are plated.

Spin Infection Protocol Section II: Spin Infection Protocol Modification

1. Remove 1 vial of frozen Retroparticles and thaw at room temperature for 10 minutes.
2. Once the virus supernatant is completely thawed, retrieve the 6-well plate containing the target cells from incubator.
3. Carefully transfer 0.5 mL of the thawed retroviral supernatant from the vial to the well containing the target cells. Gently pipet up and down to mix virus supernatant and cells. Note: it is important to avoid creating bubbles when mixing.
4. To avoid leakage of the media from the 6-well plate, carefully wrap parafilm around the edge of the 6-well plate to secure its lid is in place.
5. Carefully weigh the 6-well plate. Then set up a balance plate to the same weight by adding the same volume of medium to another 6-well plate. Secure the lid of the balance plate in place by wrapping parafilm around the edge of the plate. Note: it is important to ensure both plates have the same weight to prevent damage to the centrifuge.
6. Carefully put the plates in opposing plate holders of a swinging bucket rotor in a table top centrifuge. Spin plates at 2,500 rpm at 37°C for 90 minutes. Note: make sure the plates are secured (i.e. snugly fitted) in plate holders before commencing with spinning.
7. After the spin step is complete, carefully remove the infection plate from the centrifuge, and remove the parafilm seal. Take care not to spill the viral supernatant contained in the plate.
8. Incubate the cells at 37°C and 5% CO_2 for 5 hours.

9. After 5 hours, remove plate from the incubator, add 4 mL of complete growth medium to infected cells.
10. Incubate the cells at 37°C and 5% CO_2 for an additional 48 hours.
11. Proceed to Section IV in Detailed Protocol: Generating β-Arrestin-EA Parental Cell Lines.

Generating a Stable Clone (Optional)

While working with a β-Arrestin2-EA/GPCR-PK double stable pool is an option, generation of a clonal cell line may be desirable for three reasons:

- It may be possible to isolate a clone with a larger assay window than the stable pool
- Clonal cell lines possess superior passage stability
- It may be possible to isolate clones with more balanced expression levels of both the β-Arrestin2-EA and GPCR-PK fusion proteins

The following protocol for setting up a limiting dilution can be used to isolate a homogeneous clonal cell line from the heterogeneous stable pool. This protocol can also be used to isolate a β-Arrestin2-EA parental clonal cell line.

1. Generate a cell suspension of 2 x 10^5 cells/mL. Then serially dilute the cell suspension with 1:10 dilution ratios, in growth medium (without selection antibiotics) until a cell suspension of 20 cells/mL is achieved.
2. Seed 50 µL of cells/well into 384-well tissue culture-treated plates. As the cell suspension is being transferred to the 384-well plate, make sure the cell suspension is mixed well to ensure an even distribution of cells across the plate.
3. Carefully wrap a layer of parafilm around the edge of the plate (with the plate's lid in place). This will slow the evaporation of the media. Incubate the plate at 37°C and 5% CO_2 in a humidified incubator.
4. Incubate the plate for 6 to 8 days to allow clonal cell colonies to grow. Cell lines with slow doubling times may require more time. Then visually inspect wells to search for single colonies.
5. Mark wells that contain single colonies. These are clonal cell line candidates. Wells with multiple cell colonies should be ignored. Re-wrap the plate in parafilm and return it to the tissue culture incubator. Incubate the plate another 4 to 9 days to allow the colonies to grow larger.
6. Once the cells reach confluency in the 384-well plate, detach the cells using Trypsin-EDTA and transfer the colonies into wells of a 96-well plate. Do not combine cell colonies; each colony should be transferred to its own well on the larger plate.
7. As cells reach confluency, then transfer the cell colonies to plates with progressively larger wells: from the 96-well plate to a 48-well plate; then to a 24-well plate; then to a 6-well plate. At each of point up until it is time to transfer cells to the 6-well plate, the clone candidates should be maintained in proper growth media without selection antibiotics. Each well with cells contains a cell population that represents a clonal cell line candidate stably overexpressing the arrestin-EA and the GPCR-PK. It is recommended to add selective antibiotics (e.g. Hygromycin B and G418) to the growth media, to maintain selective pressure on both constructs, only after cells are transferred from the 24-well to a 6-well dish.
8. Once cells reach 80% to 90% cell confluency in a 24-well plate, there should be sufficient cells available to evaluate functional responses of the clone candidates (in 384-well assay plates) to an appropriate receptor agonist, while also transferring the bulk of the cells to a 6-well plate for further propagation.

a. While in the process of transferring cells from the 24-well plate to a 6-well plate, seed a portion of the cell suspension to eight wells of a 384-well assay plate. The plated cells will be used to perform a functional test of each clone candidate. Incubate the 384-well plate overnight at 37°C and 5% CO_2.

b. Treat cells with a single high dose of receptor agonist (e.g. a dose of drug which would give an 80-100% maximal response). For each clone candidate plated on the 384-assay plate, add agonist to four of the eight plated wells. Only buffer (no agonist) should be added to the other four wells.

c. Incubate the assay plate at the for the optimal time and temperature (e.g. 90 minutes at 37°C and 5% CO_2) that was determined when testing the stable pool.

d. Add PathHunter detection reagent and read the plate.

e. Calculate the Signal/Background ratios for each clone candidate.

9. Identify the functional clone candidates, and select a subset of the functionally positive clone candidates for further study. Mark the wells on the 6-well plates containing the selected clone candidates.

10. Once these grow up under selection in 6-well plate, test cells for their agonist dose responses. Use the detailed assay protocols described in the user manual for running a PathHunter β-Arrestin Assays for GPCR Cell Lines. Refer to the Protocols for Running PathHunter β-Arrestin2 Recruitment Assays in the Supplemental Information section for detailed protocols for running an agonist dose-response assay.

11. Select final clone candidates based on their dose response (e.g. correct potency and largest signal to background ratio).

12. Once clones with desirable functional characteristics are identified, it is recommended that a bank of early-passage cells be frozen down (at 1 x 10^6 cells per vial). This early-passage bank of cells can be used to start a passage stability test, and as a starting stock for generating a master working cell bank. It's recommend that the clones be tested for stability (of either function or expression) over at least 10 passages. This can be accomplished by first cryopreserving cells from multiple passages (e.g. at passages 2, 6 and 10); thawing all of the preserved cell passages at the same time; then testing: Functional stability is tested by testing cells for dose responses to agonist; expression stability can be tested using the PathHunter ProLabel®/ProLinkTM Detection Kit (Cat. No. 93-0812 Series).

Cell Freezing

The following is a procedure for freezing cells that have been cultured in T75 or T225 flasks. This protocol assumes that cells have reached 70% to 80% confluency in enough flasks to fill the desired number of cryovials, in 1 mL/vial aliquots, with the desired number of cells per vial (e.g. 1 x 10^6 per vial).

Care should be taken in handling to avoid contamination.

1. Remove T75 (or T225) flasks from incubator and place in a sterile tissue culture hood.

2. Gently aspirate the media from the flasks

3. Add 10 mL PBS into each T75 flask (or 15 mL for a T225 flask), and swirl to rinse the cells.

4. Gently aspirate PBS from the flask.

5. Add 1 mL of 0.25% Trypsin-EDTA to T75 flasks (or 3 mL to T225 flasks).
6. Gently rock the flask back and forth to ensure the surface of the flask is completely covered with Trypsin-EDTA.
7. Incubate the flasks at 37°C and 5% CO_2 for 2 to 3 minutes or until the cells have detached.
8. Remove the flask from the incubator and view under a microscope to confirm that the cells have detached. If necessary, tap the edge of the flask to detach cells from the surface.
9. Add 5 mL cell culture media containing appropriate selection antibiotic, to each T75 flask (or 15 mL to each T225 flask).
10. Using a pipette, gently rinse the cells from the surface of the flask with the added media. Gently pipette up and down several times to achieve a single cell suspension with no cell clumps.
11. Remove the entire amount of cells from the T75 flask and transfer to a 15 mL conical centrifuge tube. If using a T225 flask, transfer cells to a 50 mL conical centrifuge tube. If necessary, add additional cell culture media containing appropriate selection antibiotic, to the flasks (e.g. 3 mL for T75 flasks or 5 mL for T225 flasks), rinse to collect the remaining cells, then transfer the additional media to the conical tube. Gently pipette up and down several times to ensure a single cell suspension.
12. For the purpose of determining the concentration of cells in the suspension:
    a. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.
    b. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or another cell counting device.
    c. Count cells, calculate the concentration of cells in the suspension, and then calculate the total number of cells remaining in the 15 mL (or 50 mL) centrifuge tube.
13. Centrifuge the collected cells at 300 X g for 4 minutes.
14. After centrifugation, discard the supernatant being careful not to disturb the cell pellet.
15. Based on the total cell number calculated in Step 12 above, re-suspend cells to the desired concentration (e.g. 1 x 10^6 to 2 x 10^6 cells/mL) with ice-cold AssayComplete™ Freezing Reagent.
16. Make 1 mL aliquots by transferring 1 mL of cell suspension to labeled 2 mL cryogenic tubes; tightly cap the tubes.
17. Freeze cells in a -80°C freezer at a controlled rate (-1°C/minute) overnight. This can be performed using a dedicated cell freezer or commercially available freezing chamber (pre-chilled to 4°C). For short term storage, vials can be stored in the -80°C freezer for a maximum of two weeks.

Keep cells on ice during this process to maintain cell viability.

Frequently Asked Questions

Why is my β-Arrestin2-EA expression level low?

Reasons	Solutions
Too little or much antibiotic used for selection	Determine the antibiotic sensitivity of the target cell line by performing a kill curve experiment.
Viral stocks stored incorrectly	Viral stocks should be stored at -80°C. Do not freeze/thaw. Stock should only be used once.
Cell type difficult to transduce	Some suspension lines are refractory to infection. Perform 2 consecutive rounds of infection (2 x 1 mL of virus) before putting cells under selection to boost multiplicity of infection (MOI) if insufficient expression achieved with single infection. Additionally, refer to the Spin Infection protocol in the Supplemental Information section.
Detection reagent degraded	PathHunter detection kit contains protein components that need to be stored at -20°C. Ensure that the detection components are stored properly and they are not past their expiration date.
Cell host dependent	Some cell lines will not tolerate the overexpression of either β-Arrestin2 or the GPCR.

What factors affect cell infection efficiency?

Factor	Details
Characteristics of target cell line	The target cell lines must be dividing with a doubling time between 16 and 20 hours. Adherent cell lines are generally easier to infect than suspension cell lines.
Media content of viral supernatant	Viral supernatants are generated by harvesting spent media containing virus from the packaging cells. Spent media lacks nutrients and may contain some toxic metabolic waste products. This may affect growth of target cells.
Cells not growing optimally	Antibiotic resistance requires transgene expression. Retrovirus only stably infects replicating cells. If confluent, reduce density of the cells and replenish fresh cell growth medium prior to infection with retroparticles.
Silencing of integration site of gene	Repeat infection.

Why do my cells look unhappy after infection/transduction?

Factor	Details
Too much antibiotic used for selection	Determine the antibiotic sensitivity of the target cell line by performing a kill curve experiment.
Too many/too few target cells plated for infection; Cells not growing/ dividing optimally	Make sure target cells are plated at correct cell density. As a general rule, cells should reach confluency 72 hours after plating.
Target cells should be healthy and in log phase growth.	Viral supernatants are generated by harvesting spent media containing virus from the packaging cells. Spent media lacks nutrients and may contain some toxic metabolic waste products. This may affect growth of target cells.
Low transduction efficiency	Reduce antibiotic concentration; use conditioned media; repeat transduction and increase target cell density (e.g. reduce the well size/media volume so cells can condition their media).

For additional information or technical support, please contact technical support at SupportUS@discoverx.com or SupportEurope@discoverx.com

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